Functional and Biochemical Properties of the Hemoglobins of the Burrowing Brittle Star *Hemipholis elongata* Say (Echinodermata, Ophiuroidea)

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Abstract: The burrowing brittle star *Hemipholis elongata* (Say) possesses hemoglobin-containing coelomocytes (RBCs) in its water vascular system. The RBCs, which circulate between the arms and body, are thought to play a role in oxygen transport. The hemoglobin of adult animals has a moderate affinity for oxygen ($P_{50} = 11.4$ mm Hg at pH 8, 20 °C, measured *in cellulo*) and exhibits cooperativity (Hill coefficient > 1.7). The hemoglobin of juveniles has a higher affinity ($P_{50} = 2.3$ mmHg at pH 8.0, 20 °C) and also exhibits cooperativity. The oxygen-binding properties of the hemoglobin are relatively insensitive to pH, temperature, and hydrogen sulfide. Adult hemoglobin is a heterogeneous mixture composed of three major fractions. The combined results of electrospray mass spectrometry and oxygen-binding experiments performed on purified fractions indicate that the native hemoglobin is in the form of homopolymers. A partial amino acid sequence (about 40 amino acids) of adult hemoglobin reveals little homology with holothurian hemoglobins.

Introduction

The hemoglobin of the burrowing brittle star *Hemipholis elongata* Say (Echinodermata, Ophiuroidea) is contained in anucleate coelomocytes (red blood cells, RBCs) present in the water vascular system (WVS) (Hajduk and Cosgrove,

1975; Hajduk, 1992; unpubl. data). The presence of RBCs in the WVS imparts a bright red color to the tube feet, which are external projections of the WVS, and readily distinguishes *H. elongata* from other burrowing ophiuroids (family Amphiuridae) occurring in the same locations. RBCs and the fluid of the WVS are circulated throughout the body by a series of synchronous contractions of the tube feet (Beardsley and Colacino, 1998). *H. elongata* does not ventilate its burrow, and it has been hypothesized that hemoglobin in the WVS transports oxygen from arms extended into the water column to buried body parts (Beardsley and Colacino, 1998).

H. elongata is often found in the lower intertidal zone in protected, low-energy areas of the southeast coast of the United States (Hendler *et al.*, 1995), and the sediments it inhabits are often soft, poorly oxygenated and may contain hydrogen sulfide (Windom and Kendall, 1979; Camargo, 1982; unpubl. data). The distribution of *H. elongata* is sporadic, but densities in a given area may be as high as 2000/m^2 (Valentine, 1991a). The juveniles of *H. elongata* often settle out onto the arms of the adults (adult disc diameter 5 to 12 mm) (Mortensen, 1920; Valentine, 1991a, b), crawl down the arms of the adult, and grow in the burrow until they reach a size at which they can establish their own burrows. Recently settled juveniles (disc diameter ≥ 0.48 mm) also possess RBCs. Although juveniles smaller than 0.43 mm apparently do not have RBCs (unpubl. data), the stage at which they start producing RBCs is unknown.

Only three of the approximately 2000 species of brittle stars possess hemoglobin: *Ophiactis virens* (Foettinger,

1880; Cuenot, 1891), *Hemipholis elongata* (Hajduk and Cosgrove, 1975; Heatwole, 1981; Beardsley *et al.*, 1993), and *Ophiactis simplex* (Christensen, 1998). Of these three, only *H. elongata* burrows; the other species inhabit the fouling communities of rock jetties and pilings. As for the functional and biochemical properties of ophiuroid hemoglobins, very little has been reported in the literature (Hajduk and Cosgrove, 1975; Beardsley *et al.*, 1993; Christensen, 1998; Weber and Vinogradov, 2001). Hajduk and Cosgrove (1975) reported that the hemoglobin of *H. elongata* has a high affinity for oxygen ($P_{90} = 9$ mmHg) and is composed of five components separable by acrylamide gel electrophoresis. They also reported two components with molecular weights of 19,000 and 23,000 Da separable by SDS gel electrophoresis. The hemoglobins of the holothurians, the other group of extant echinoderms possessing the iron-containing pigment, have been more thoroughly investigated (Terwilliger and Terwilliger, 1988 [review]; Suzuki, 1989; Mauri *et al.*, 1991; McDonald *et al.*, 1992; Baker and Terwilliger, 1993; Kitto *et al.*, 1998).

The goal of the present study was to characterize the functional, biochemical, and structural properties of the hemoglobins of *Hemipholis elongata* in more detail. Comparisons were made with the findings of Hajduk and Cosgrove (1975), and the functional and structural properties observed in this study were compared to those of the holothurian hemoglobins.

Materials and Methods

Collection and care of animals

Animals were collected with a shovel and sieve during low tide from Johnson Creek, Hunting Island, South Carolina. Animals were transported to the laboratory and kept at room temperature (24 °C) in an aquarium containing sediments from the collection site and aerated natural seawater. All experiments using whole animals or RBCs were conducted within 6 weeks of collection. Unless otherwise noted, all experiments used hemoglobin or RBCs from adult animals.

Preparation of hemolysates and determination of total hemoglobin per animal

RBCs were extracted by cutting the animal into small fragments in a small quantity of buffered seawater (50 m*M* TRIS, pH 8.0 at 20 °C) and then rinsing the fragments until no red color was visible. The body fragments were removed and the cell/buffer mixture was placed on ice. The cells were washed three times in fresh buffer. After a final wash, the supernatant was removed and the cell pellet frozen and thawed to lyse the cells. The thawed pellet was resuspended in about 1 ml of 50 m*M* TRIS, pH 8 at 20 °C in distilled water and centrifuged at 14,000 × *g* for 5 min to remove

cell remnants and debris. The hemolysate was diluted with appropriate buffer to an absorbance of 0.4 to 0.5 OD units at 540 nm to minimize photometric error (van Assendelft, 1970).

For determination of total hemoglobin, the absorption spectrum of the solution was recorded in a Beckman DU-65 spectrophotometer. The hemoglobin concentration, as heme, was calculated using the Beer-Lambert law and the extinction coefficient for human hemoglobin at 542 nm (van Assendelft, 1970). This concentration was multiplied by the total sample volume to give total hemoglobin (as heme) in millimoles, and then divided by animal wet weight to obtain total hemoglobin per gram of wet weight.

Hemolysates used in the oxygen-binding equilibria and the sulfide sensitivity experiments were prepared as above, but with RBCs taken only from excised arms with the aim of avoiding potential interfering effects due to enzymes released from the gut and gonads. Such material may have increased the formation of methemoglobin in the whole animal hemolysates (see Discussion for methemoglobin effects).

Intracellular heme concentration

RBCs collected from an animal as described above were resuspended in isotonic filtered seawater buffered to pH 8.0 at 20 °C with 50 m*M* TRIS. A few drops of a suspension of polystyrene microbeads (Polysciences, Inc.) of 5.85 µm ± 0.13 µm diameter were added to the cell suspension. A small drop of this mixture was placed on a glass microscope slide, and a glass coverslip was placed on top of the drop. Excess water was wicked away with a tissue until the coverslip was resting on the microbeads, and the cells were flattened between the coverslip and the slide. This technique serves to set the pathlength for the subsequent absorbance measurements (Colacino and Kraus, 1984). The slide was then placed on the stage of the microspectrophotometer (Mangum *et al.*, 1989). The light transmission spectrum through an area of the slide containing only buffer was collected as a reference. Cells were picked at random on the slide, and the transmission spectrum was recorded through each. The absorbance at 540 nm, 560 nm, and 577 nm was computed from light transmission data. The intracellular hemoglobin concentration was calculated as heme concentration using the Beer-Lambert Law and the extinction coefficients for human hemoglobin at the chosen wavelengths (van Assendelft, 1970). Concentrations calculated at the three wavelengths were averaged for each cell. A total of 48 cells were measured (12 cells on each of four slides prepared from the same cell suspension).

Separation of hemoglobins

The presence of multiple hemoglobins was determined using a Pharmacia fast protein liquid chromatography

(FPLC) system. Crude hemolysates were collected as previously described and resuspended in 50 mM TRIS, pH 8.0, at 20 °C. Hemolysates from 7–10 individuals were pooled due to the small quantities of hemoglobin collected from each animal. The pooled samples were equilibrated with 50 mM TRIS, pH 8.0, by dialysis. The hemolysates were then loaded onto a DEAE Q Sepharose Hi-Load 2610 column, 60-ml column volume. The resin had been equilibrated with four volumes of 50 mM, pH 8.0, TRIS buffer. A linear salt gradient (50 mM TRIS to 0.075 M NaCl + 50 mM TRIS) was used for elution. The total gradient volume was 300 ml. Fractions were collected at a rate of 6 min per fraction, and absorbance was read at 280 nm (for protein) and 415 nm (hemoglobin peak).

The fractions showing peak absorbance were electrophoresed on a Pharmacia Phast system using a native gel. Samples were run on a 10% acrylamide gel with pH 8.3 electrophoresis buffer and stained with Coomassie blue. A low-porosity stacking gel (upper fraction) was used to sharpen the banding pattern. Samples contained 5–25 μg of protein. Crude hemolysate and human hemoglobin A were electrophoresed as references.

Determination of molecular weight

The molecular weights of the purified hemoglobin fractions were determined by electrospray ionization mass spectrometry. Samples were prepared by the method described by Stevens *et al.* (1994). Measurements were made on a Fissons-VG BIO-Q triple quadrupole mass spectrometer equipped with a pneumatically assisted electrospray ionization source operating at atmospheric pressure (supplied by VG Biotech, Altrincham, UK).

Hemoglobin spectra and oxygen-binding equilibria in cellulo

A small portion ($\leq$ 5 mm) of the distal end of an arm was excised from an unanesthetized animal and rinsed in buffer to remove any adhering mud. The arm tip was placed in a large depression slide containing a small amount of 0.45-μm filtered isotonic seawater buffered with 50 mM TRIS of the desired experimental pH (7, 8, or 9). The tube feet were manually stimulated to contract, forcing the RBCs out of the cut end of the radial canal. About 50 μl of the buffer/RBC mixture was transferred to a specially designed gas slide (Colacino and Kraus, 1984). The gas slide was placed on the microscope stage of a diode array microspectrophotometer (Mangum *et al.*, 1989). The slide was maintained at a constant temperature (20° $\pm$ 0.5 °C) with water pumped from a refrigerated water bath (Forma Scientific). The internal gas tension of the slide was controlled by a gas-mixing flowmeter (Cameron Instrument Co.). The gases were humidified and brought to experimental temperature before flowing through the sample chamber of the gas slide

at 100 ml/min. A total of 60 cells taken from 47 animals were used in these experiments.

To examine the equilibrium oxygen-binding characteristics of the hemoglobin *in cellulo*, cells were exposed to gases at nine oxygen tensions, ranging from 0 to > 150 mmHg (room air). Fractional saturation values were computed from transmitted light intensities (540 nm, 560 nm, and 580 nm) using a two-wavelength modification of a standard analysis (Rossi-Fanelli and Antonini, 1958). The effect of pH on oxygen affinity was determined from oxygen equilibrium experiments conducted at pH 7.0, 8.0, and 9.0. Temperature effects on oxygen binding were determined from oxygen affinity experiments conducted at 10 °C, pH 8.0, and 20 °C, pH 8.0. The heat of oxygenation (ΔH) was calculated from the van't Hoff equation.

Oxygen-binding equilibria in vitro

For measurements on crude hemolysates, about 50 μl of the hemolysate was placed in the working chamber of the gas slide along with a 0.5-cm^2 piece of monofilament nylon mesh (105-μm mesh opening) (Small Parts, Inc.). A freshly prepared hemolysate solution was used in each experiment because repeated freezing and thawing increased methemoglobin formation (see Discussion for methemoglobin effects). The nylon mesh served to ensure a stable pathlength for light transmission measurements. Oxygen-binding equilibria were measured as previously described for *in cellulo* measurements at 20 °C. Measurements on purified FPLC fractions were made using standard tonometric techniques at pH 7 at 20 °C.

Oxygen-binding equilibria of juvenile hemoglobin

Oxygen-binding equilibria for hemoglobins of juvenile *H. elongata* were measured on both isolated cells and intact animals. For the isolated-cell measurements, cells were collected from individuals of disc diameter $\leq$ 1 mm (n = 5) by excising a small portion of an arm in a small quantity of buffer, 50 mM TRIS, pH 8.0, at 20 °C. The cells were loaded onto the gas slide, and the oxygen binding was measured by the method previously described for the adult cells.

For the whole-animal measurements, an intact juvenile was loaded onto the gas slide. The juvenile was anesthetized by placing it in a small quantity of buffer (< 200 μl) containing a few drops of 7% MgCl$_2$. The individual was then placed centrally in the gas slide chamber in a small drop of buffer/MgCl$_2$. Hemoglobin spectra were taken through a tube foot with RBCs in it. Oxygen-binding experiments were conducted as before. Four individuals were measured in this manner.

Hemoglobin sensitivity to sulfide

The effects of hydrogen sulfide on oxygen-binding equilibria were examined using hemolysates prepared as described earlier (hemoglobin 50 mM TRIS in distilled water, pH 8.0 at 20 °C, adjusted to give an absorbance reading of 0.4–0.5 at 540 nm in an 0.5-cm cuvette). Four milliliters of this solution were placed into each of two glass tonometers equipped with 0.5-cm pathlength cuvettes. The initial oxygenated spectra were recorded from 650 nm to 400 nm on a Beckman DU 65 spectrophotometer. The samples were deoxygenated with 99.999% N$_2$, and a deoxygenated spectrum was taken. Then 100 μl of 10 mM Na$_2$S solution was injected into one tonometer with a syringe; 100 μl of deoxygenated buffer was added to the control tonometer. The spectrum was again recorded. Oxygen was gradually introduced in a stepwise fashion by the injection of room air samples after an equal volume had been removed from the tonometers, and the spectra were measured after a 10-min equilibration. This experiment was repeated three times with freshly prepared hemolysates pooled from three to five individuals.

Stopped-flow measurements of ligand kinetics

Kinetics of the hemoglobin-oxygen reaction were estimated using the stopped-flow technique on the crude cell hemolysates suspended in 50 mM HEPES, pH 7, at 20 °C; the crude hemolysates represent pooled samples. Measurements were made for the O$_2$ "off" reaction (dissociation) and the CO "on" reaction (association). The CO "on" reaction was also examined using flash photolysis. The presence of modulator effects was determined by performing the above reactions in the presence of ATP.

Experiments were performed on a Gibson-Durrum stopped-flow apparatus that consists of a Durrum model 13000 light source and monochromator and Durrum model 110 stopped-flow instrument with pneumatic drive. The dissociation constant, k_{off}, was determined by reacting oxyhemoglobin, from crude hemolysates, with sodium dithionite (Na$_2$S$_2$O$_4$) (about 0.5%). The time course of the reaction was monitored by measuring transmitted light intensities.

Light intensity data were collected by a DASAR data acquisition, storage, and retrieval system with a DW-2 interface to a Tektronix 4052 computer. Initial data analyses were carried out with the ASYST program (Macmillan Software Co.) prior to curve-fitting analysis by a nonlinear least-squares program (Johnson *et al.*, 1981).

The carbon monoxide (CO) association reaction was monitored by the stopped-flow technique and flash photolysis. Flash photolysis was performed with dual fast extinguishing (approximately 30 μs) flash tubes and a Xenon Corp. model B micropulser. The subsequent association of CO and the hemoglobin was then monitored as before.

Amino acid sequence of hemoglobin fractions

Partial amino acid sequences of each purified hemoglobin fraction were determined by the automated Edman method using a Porton Instruments PI 2090 integrated micro-sequencing system. The amino acids were identified by visual inspection of hardcopy plots, using retention times from a standard of PTH amino acids.

Proteins were not digested with proteases prior to sequencing. Each analysis used 150–200 pmol of protein. Samples of fractions 1 and 2 were processed for 40 cycles, at which time it became difficult to distinguish the amino acid peaks from the background noise. Fraction 3 was run for 21 cycles.

The partial sequence for fraction 1 was compared to other protein sequences using the MacVector sequence analysis software (Oxford Molecular Group PLC) and the *Entrez* database (National Center for Biotechnology Information).

Results

Total hemoglobin per individual

The average wet weight of the adult animals used for this measurement was 0.39 g $\pm$ 0.16 g (mean $\pm$ SD, $n = 9$). These individuals contained 8.5 $\times$ 10^{-5} mmol $\pm$ 4.4 $\times$ 10^{-5} mmol of hemoglobin, measured as heme. Using a hemoglobin subunit molecular mass of 16,000 Da, this figure translates into 0.35% of the total body mass accounted for by the hemoglobin.

Intracellular hemoglobin concentration and estimation of hematocrit

The RBC hemoglobin concentration, as heme, was 19.5 $\pm$ 5.0 mM (mean $\pm$ SD, $n = 48$ cells). Using a mean cell diameter of 9 μm (Hajduk and Cosgrove, 1975; Heatwole, 1981; unpubl. data) and an intracellular hemoglobin concentration of 19.5 mM, the volume of a single cell is 3.8 $\times$ 10^{-7} mm^3 and contains 7.4 $\times$ 10^{-12} mmol hemoglobin. If an animal has a total of 8.5 $\times$ 10^{-5} mmol of hemoglobin, it has approximately 1 $\times$ 10^7 cells. The total cell volume is equal to 4.3 mm^3; and for an animal with a total WVS volume of 18.1 mm^3 (Beardsley and Colacino, 1994), the fraction of the WVS taken up by cells is 0.24.

Separation of the hemoglobins

The hemoglobin is a heterogeneous mixture. Separation of oxygenated crude cell hemolysates by DEAE Q Sepharose FPLC resulted in three fractions that absorb at 280 and 415 nm (Fig. 1). Native gel electrophoresis of the crude hemolysates yielded five bands, two of the major bands corresponding to FPLC fractions 1 and 2, a third major band of uncertain identity, and two minor bands (Fig. 2). Lack of
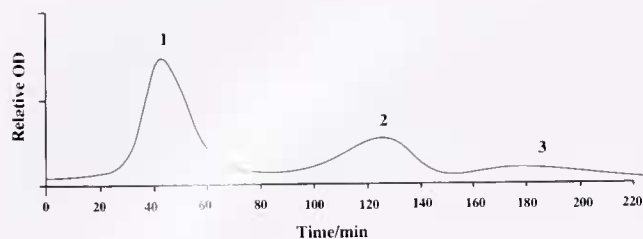
Figure 1. Chromatogram of crude hemolysates from *Hemipholis elongata*, separated by FPLC ion exchange chromatography. Separation was achieved using a DEAE Q Sepharose Hi-Load 2610 column, 60-ml column volume. A linear salt gradient was established from 50 mM Tris to 0.75 M NaCl + 50 mM Tris; gradient volume was 300 ml. The profile represents absorbance at 280 nm and 415 nm.

banding in lane 5 (FPLC fraction 3) is attributed to low concentration applied to the gel.

As the sample placed on the column was a pooled sample, it is unknown if all of the FPLC fractions are found in a single individual or in separate individuals. However, the separation was performed on two occasions using animals from different collections, and both experiments yielded the same results.

Molecular weight of hemoglobin subunits

The technique used to determine molecular weight, electrospray mass spectrometry, breaks polymeric hemoglobins into monomeric subunits and causes the heme to dissociate from the protein. The signals for FPLC fractions 1 and 2 indicated that each was composed of a single protein and a heme peak. The molecular weights of the subunits were as follows: fraction 1—16,080, fraction 2—16,119, and fraction 3—16,143. The hemes all had a molecular weight of 616. The differences in the protein weight indicate differences in amino acid composition for the three subunits.

The mass spectrum generated by the third FPLC fraction indicated the possibility of more than one subunit. However, the low concentration of this sample resulted in high-noise mass spectrometry data, making this conclusion uncertain.

Oxygen-binding equilibria in cellulo and in vitro

The hemoglobin of *Hemipholis elongata* has a moderate affinity for oxygen (P_{50} = 11.4 mmHg at pH 8.0, 20 °C) (Table 1). The P_{50} at pH 8.0 is similar to those reported for holothurian hemoglobins (Table 1). The Hill numbers (n) were greater than 1 for both *in cellulo* and *in vitro* measurements, indicating cooperativity and functional hemoglobin composed of at least two subunits (a dimer) (Table 1). Then n values are greater for the *in cellulo* measurements (n = 2.81) than for the crude hemolysates (n = 1.91). The difference in Hill coefficients may be due to concentration differences (19.5 mM *in cellulo* vs. 0.1 mM *in vitro*) (see Discussion for concentration effects).

The P_{50} values measured on the FPLC fractions are lower than those measured on the crude hemolysates and *in cellulo*. P_{50} at pH 7.0 at 20 °C for fraction 1 is 6.1 mmHg and that for fraction 2 is 2.5 mmHg. These low values may be due to the formation of methemoglobin (see Discussion). The Hill numbers for the purified FPLC fractions 1 and 2 are greater (about 1.8) (Table 1).

Values for P_{50} measured at the three pHs are significantly different from one another (Student's t test, P = 0.05) (Table 1). Oxygen affinity increases slightly as pH decreases. This is opposite to the usual pH effect. The slope of the Bohr plot is 0.072. The temperature dependence of the oxygen affinity is small (P_{50} = 8.9 mmHg at 10 °C vs. 11.4 mmHg at 20 °C). The heat of oxygenation (ΔH) is −4.1 kcal/mol.

Oxygen-binding equilibria of juvenile hemoglobin

The hemoglobin of juveniles (disc diameter 0.48 to 0.8 mm) has a higher affinity (P_{50} = 2.3 mmHg *in cellulo* and 4.0 mmHg in tube feet of intact animals, pH 8.0 at 20 °C) for oxygen than the adult hemoglobin (P_{50} = 11.4 mmHg, disc diameter 5 to 12 mm) (see Table 1). This was true for both isolated RBCs and intact animal measurements. The greater apparent P_{50} for measurements using intact animals may be explained by oxygen consumption of the animal. The Po_2 within the tissues is lower than the external Po_2 due to oxygen consumption by the tissues. The difference in Po_2 can lead to an overestimation of the P_{50}. Even with the

1 2 3 4 5

Figure 2. Native gel electrophoresis of crude hemolysates and purified FPLC fractions of *Hemipholis elongata* hemoglobin. Samples were run on a 10% acrylamide gel with pH 8.3 electrophoresis buffer and stained with Coomassie blue. A low-porosity stacking gel (upper fraction) was used to sharpen the banding pattern. Lack of banding in lane 5 is attributed to low concentration applied to the gel, since mass spectrometry results showed fraction 3 to contain hemoglobin. Lane 1: Human hemoglobin A; lane 2: Crude hemolysate; lane 3: FPLC fraction 1; lane 4: FPLC fraction 2; lane 5: FPLC fraction 3.

Table 1

Oxygen P_{50} values and Hill numbers of echinoderm hemoglobins (mean $\pm$ SE)

Species	P_{50} mmHg	Hill number (n)	# of measurements (# of individuals)*	Study
Ophiuroids				
Hemipholis elongata				
in cellulo				Present study
pH 7.0, 20 °C	9.5 ± 1.2	2.35 ± 0.07	15 (11)	
pH 8.0, 10 °C	8.9 ± 1.1	3.20 ± 0.10	12 (8)	
pH 8.0, 20 °C	11.4 ± 1.2	2.81 ± 0.08	17 (14)	
pH 9.0, 20 °C	13.1 ± 1.2	2.67 ± 0.09	16 (14)	
in vitro crude hemolysates				Present study
pH 7.0, 20 °C	8.2 ± 1.1	1.78 ± 0.04	6	
pH 8.0, 20 °C	10.5 ± 1.1	1.91 ± 0.05	4	
pH 9.0, 20 °C	11.3 ± 1.2	1.73 ± 0.06	4	
in vitro purified				Present study
fraction 1, pH 7.0, 20 °C	6.1	1.83	2	
fraction 2, pH 7.0, 20 °C	2.5	1.41	2	
juvenile				Present study
in vitro, pH 8.0, 20 °C	4.0 ± 1.2	3.28 ± 0.37	4	
in cellulo, pH 8.0, 20 °C	2.3 ± 1.5	1.76 ± 0.51	5 (4)	
Ophiactis simplex in cellulo, pH 8.0, 20 °C	22.3 ± 1.2	3.04 ± 0.18	15 (12)	Christensen, 1998
Holothuroids†				
Cucumaria miniata	8.0 ± 1.5	1.86 ± 0.07		Terwilliger, 1975
Cucumaria curata	7.1	1.6		Roberts et al., 1984
Thyonella gemmata	2.6	1.4		Steinmeier & Parkhurst, 1979
Molpadia oolitica	4.0	1.6		Terwilliger & Read, 1972
Molpadia intermedia	2.0	<1.0		Manwell, 1966
Caudina arenicola	3.5	1.5		Bonaventura et al., 1976
Sclerodactyla (Thyone) briareus	8.1	1.08		Colacino, 1973
Paracaudina chilensis	1.5	1.3		Baker & Terwilliger, 1993

* The numbers in # of measurements reflect the number of cells measured; the number in parentheses reflects the number of individuals represented in the experiment. No animal was used more than twice.

† All holothurian P_{50} values were measured *in vitro*.

overestimation of the juvenile P_{50}, the value is significantly less than that of the adult, indicating distinct functional differences.

Hemoglobin sensitivity to sulfide

Exposure to sulfide caused no changes in the absorption spectrum, and there was no peak at 620 nm; this peak is characteristic of human sulfhemoglobin in the visible region of the spectrum (van Assendelft, 1970; Carrico et al., 1978). There was also no change in the oxygen affinity of the hemolysates in the presence of sulfide (Fig. 3).

Kinetics of ligand binding

Both of the ligand reactions with the crude hemolysates were biphasic, with the two components in each reaction accounting for about 50% of the reacting species (134.6 ± 2.2 (SD) s^{-1} and 22.3 ± 0.3 s^{-1} for the O$_2$ "off" reaction and 5.2 × 10^4 M^{-1}s^{-1} and 2.8 × 10^4 M^{-1}s^{-1} for the CO "on" reaction). These data are consistent with the heteroge-

neity of the crude hemolysate. Given that FPLC reveals three distinct hemoglobins with two (fractions 1 and 2) accounting for the majority of the protein, one might expect to see two distinct reaction components in ligand-binding experiments. A third kinetic component representing the third FPLC fraction was not seen, either due to the small amount of it present in the crude hemolysates or to a lack of difference in rate constants.

There was no effect of ATP on the CO "on" reaction. However, the presence of ATP caused a small, but statistically significant ($P = 0.05$) increase in the dissociation rate constant for both phases of the biphasic oxygen "off" reaction of *H. elongata* hemoglobin (144.3 ± 2.4 s^{-1} and 24.2 ± 0.2 s^{-1}).

Amino acid sequence of the protein

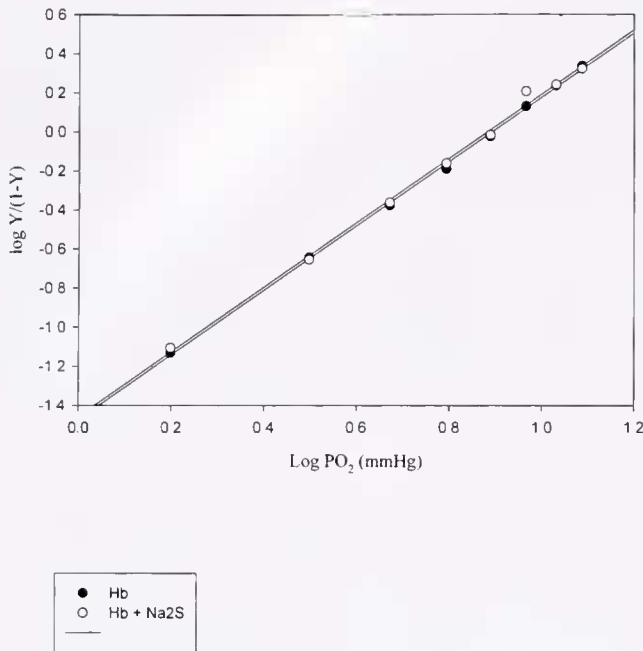Figure 4 shows the partial amino acid sequences for the three FPLC fractions of the hemoglobin. The sequences for

Figure 3. Hill plots for the oxygen-binding equilibria of *Hemipholis elongata* hemoglobin in the absence (●) and presence of Na₂S (○). Hemoglobin is crude hemolysate suspended in 50 m*M* Tris, pH 8.0, at 20 °C.

fractions 1 and 2 differ by eight amino acids. The partial sequence for fraction 3 was identical to that of fraction 2.

Neither hemoglobin fraction appears to be blocked at the N-terminus of the globin, unlike the hemoglobins of many holothurians (Terwilliger and Terwilliger, 1988). Proteins blocked at the N-terminus are resistant to the Edman reaction used in protein sequencing (Kitto *et al.*, 1976). No modification (*e.g.*, digestion with proteases) of the *H. elongata* protein was necessary to obtain amino acid sequences.

Discussion

Intracellular hemoglobin concentrations and hematocrit

The intracellular heme concentration of *Hemipholis elongata* (19.5 m*M*) is comparable to the values reported for

holothurian RBCs (12.5 m*M* for *Sclerodactyla (Thyone) briareus* [Colacino, 1973] and 15.8 m*M* for *Cucumaria miniata* [from data in Manwell, 1959, and Terwilliger and Read, 1972]). These values are similar to those of human RBCs (20 m*M* heme) (calculated from Guyton, 1991) and phoronid (*Phoronis architecta*) RBCs (14 m*M* heme) (Vandergon and Colacino, 1989). If the hemoglobin of *H. elongata* exists as dimers, this would mean that the intracellular hemoglobin concentration is 9.8 m*M*.

The computed hematocrit value of *H. elongata* (0.24) is comparable to the tube foot hematocrits of the holothurian *S. briareus* (0.24, by direct measurement) (Colacino, 1973). These values are greater than the reported hematocrits of the perivisceral fluids of the holothurians *Cucumaria pseudocurata* (0.032) (Roberts *et al.*, 1984) and *Paracaudina chilensis* (0.015) (Baker and Terwilliger, 1993), but less than those for humans (0.4) (Guyton, 1991) and other mammals (≥ 0.4) (Schmidt-Nielsen, 1990).

Structural properties of the hemoglobin

The hemoglobins of *H. elongata* are a heterogeneous mixture of three components, as evidenced by FPLC. Because the samples were pooled from several individuals, it is not known whether hemoglobin heterogeneity is a normal characteristic of *H. elongata* blood or an artifact of sample mixing. However, the identical results (*e.g.*, same ratio of fractions) obtained on two separate occasions suggest that all three fractions are present within an individual. Hajduk and Cosgrove (1975) reported only two components separable by gel filtration, one apparently composed of monomers and the other of dimers. The difference in number of fractions may be attributable to difference in separation techniques. Gel filtration separates on the basis of size and shape; DEAE Q Sepharose FPLC (ion exchange) separates molecules on the basis of charge.

Both the present study and Hajduk and Cosgrove (1975) obtained five bands by acrylamide gel electrophoresis. Two of the major bands reported here correspond to FPLC fractions 1 and 2. Because this sample was a crude hemolysate, the two minor bands may have been due to proteins other

F1:	val	ile	ser	ala	**gly**	glu	lys	**thr**	leu	ile	arg	**asp**	ser	trp	**ala**	**pro**	val	tyr	**ala**	gly	asp
F2:	val	ile	ser	ala	**asp**	glu	lys	**asn**	leu	ile	arg	**ser**	?	trp	**phe**	**thr**	val	tyr	**ser**	gly	asp
F3:	val	ile	ser	ala	asp	glu	lys	asn	leu	ile	arg	ser	?	trp	phe	thr	val	tyr	ser		

Fractions, continued

F1:	arg	phe	gln	**ile**	gly	val	**asn**	val	phe	thr	asn	phe	?	?	ala	tyr	pro	ala
F2:	arg	phe	gln	**val**	gly	val	**asp**	val	phe	thr	asn	phe	?	?	ala	tyr		

Figure 4. Partial amino acid sequences for the three FPLC fractions of *Hemipholis elongata* hemoglobin. Amino acids in bold type represent differences in primary sequence;? represents amino acids that could not be definitively identified. F1 = fraction 1, F2 = fraction 2, F3 = fraction 3.

than hemoglobin, found within or associated with the RBCs. Due to the presence of heme at high relative concentration, two of the major bands from the crude hemolysate were visible on the gel prior to staining with Coomassie blue. The two minor bands were not visible. If the minor bands do represent hemoglobins, the low concentration, as evidenced by the faintness of the bands on the gel, could explain why these fractions were not isolated by FPLC.

The molecular masses of the various fractions (approximately 16,000 Da) are comparable to human β chain (15,860 Da) (Dickerson and Geis, 1980) and holothurian hemoglobin monomers (17,000–18,000 Da) (Terwilliger and Terwilliger, 1988). These values are smaller than those reported by Hajduk and Cosgrove (1975) (19,000 and 23,000 Da). Differences between the previously reported weights and those of the present study may be attributed to differences in techniques. Mangum (1992) remarked that many of the early studies on holothurian hemoglobin reported similarly large molecular weights that were later found to be smaller (~17,000 Da). She attributed these differences to refinement of molecular techniques.

The cooperative binding of oxygen, both *in cellulo* and *in vitro* (Hill number $\geq$ 1), indicates that the functional hemoglobin exists as a polymer. Many of the holothurian hemoglobins are known to exist as dimers (Terwilliger and Terwilliger, 1988) and possibly tetramers (Baker and Terwilliger, 1993). The purified fractions, 1 and 2, of *H. elongata* hemoglobin have only one globin chain type but exhibit cooperativity in oxygen binding, suggesting that both are able to form cooperative homodimers. The Hill numbers typically observed at low concentrations, somewhat less than 2, are consistent with the formation of cooperative homodimers. Larger assemblies may form under some circumstances, as evidenced by Hill numbers greater than 3 that were observed under some conditions (*e.g.*, *in cellulo*).

The normally existing homopolymers of several invertebrate hemoglobins exhibit cooperativity (*Scapharca inaequivalvis* [Chiancone *et al.*, 1981; Royer *et al.*, 1985]; holothurians [Terwilliger and Read, 1972; Bonaventura *et al.*, 1976]), although homodimers and homotetramers of human (and other vertebrate) hemoglobins do not. In fact, the mechanism for cooperativity in invertebrates is thought to be different from that utilized by vertebrates (Riggs, 1998). Cooperativity is thought to be due to interactions between the E and F helices of the hemoglobin subunits, first described in the arcid clam *Scapharca inaequivalvis* (Royer *et al.*, 1985, 1990). This same association has been described for the innkeeper worm, *Urechis caupo* (Kolatkar *et al.*, 1994) and a sea cucumber, *Caudina arenicola* (Mitchell *et al.*, 1995). However, Kitto *et al.* (1998) believe that the cooperativity mechanism in *C. arenicola* differs from that in *S. inaequivalvis* as the residues involved at the crucial contact points are different for the two species. Further

structural studies on *H. elongata* hemoglobin are needed to investigate the nature of the interactions of its subunits.

Comparison of the amino acid sequence of fraction 1 from *H. elongata* with sequences reported for the globins of the holothurians *Caudina arenicola* (Mauri *et al.*, 1991; McDonald *et al.*, 1992) and *Paracaudina chilensis* (Suzuki, 1989) reveals little homology. The lack of homology between the ophiuroid and holothurian globins has contributed to the inability to identify the brittle star globin gene by using holothurian primers (Kitto, pers. comm.). Furthermore, no successful primers for the hemoglobin gene have been generated based on the 39 amino acid sequence of the ophiuroid (Kitto, pers. comm.).

Oxygen-binding characteristics of the hemoglobin

The hemoglobin of *H. elongata* has a moderate affinity for oxygen, both *in cellulo* and *in vitro*. It is not certain at this time whether all of the FPLC fractions exist in the same cell, in separate cell populations, or even within the same animal. However, a large number of cells from many individuals were examined microspectrophotometrically and little variation was seen within the measured P_{50} values observed within each treatment. This suggests that the different hemoglobins represented by the purified FPLC fractions are present within a single cell. The ratio of this mixture is unknown.

The oxygen affinity results of the present study differ greatly from those reported by Hajduk and Cosgrove (1975) (P_{90} = 9 mmHg). On the basis of the Hill numbers from the present study, a hemoglobin with a P_{90} of 9 mmHg (pH 7.2) should have a P_{50} of about 3 mmHg. The *in cellulo* and *in vitro* analysis in the present study demonstrated a P_{50} between 8 and 9 mmHg at pH 7.0, 20 °C. This difference in oxygen affinities may be attributable to the formation of methemoglobin. The oxygen affinity of mammalian hemoglobin increases with increasing percentages of methemoglobin in the sample (Darling and Roughton, 1942)

Several of the holothurian hemoglobins are prone to oxidization and denaturation at pH 7.0 (Terwilliger and Read, 1972; Bonaventura *et al.*, 1976; Steinmeier and Parkhurst, 1979). When the crude hemolysates of *H. elongata* were repeatedly frozen and thawed, the P_{50} decreased. Tests for the presence of methemoglobin showed an increase in the amount present in the sample. Freshly prepared *H. elongata* hemolysates were typically 5%–7% methemoglobin as determined by the ferrocyanide method (van Assendelft, 1970). In one experiment, the methemoglobin fraction was 13% initially and rose to 45% by the end. The P_{50} calculated for this run was 3.6 mmHg. This corresponds to the P_{50} value estimated from the data of Hajduk and Cosgrove (1975).

The formation of methemoglobin may explain the low P_{50} of the purified fractions. Although it is not unusual for

different hemoglobins within an individual to have different binding affinities, the apparent P_{50} of the mixture usually lies between those of the separate fractions, not above them. During oxygen-binding experiments on the purified fractions, methemoglobin rose from 10% to 16% of the total in fraction 1 and from 32% to 42% in fraction 2. While the large proportion of methemoglobin, particularly in fraction 2, makes comparisons of P_{50} difficult, the importance of this experiment is the demonstration of the cooperative binding of homopolymers.

Another explanation for the difference in oxygen affinities may be the presence of intracellular modulators that would have been removed during the purification of the fractions. However, many invertebrate hemoglobins, including the holothurian hemoglobins, are insensitive to organic phosphates (Terwilliger and Terwilliger, 1988; Scholnick and Mangum, 1991; Baker and Terwilliger, 1993). The rate constant for the oxygen "off" reaction of *H. elongata* crude hemolysates exhibited a small, but significant, increase in the presence of ATP.

The differences in apparent cooperativity between the *in cellulo* and *in vitro* measurements may be due to concentration effects. Dilute preparations may be less aggregated than concentrated ones, with the consequence that the Hill coefficient increases due to subunit interactions as the concentration of hemoglobin increases. This may account for the fact that the hemoglobins of capitellid worms were reported to have greater cooperativity *in cellulo* than *in vitro* (Mangum *et al.*, 1992).

Other than those reported here, no data on ligand-binding kinetics are available for the brittle star hemoglobins. The smaller of the two dissociation rate constants for *H. elongata* hemoglobin is similar to those of human ($\sim$40 s^{-1}, Antonini and Brunori, 1971) and holothurian hemoglobin (*S. briareus*, 4.8 s^{-1} [Colacino, 1973]; *Thyonella gemmata*, 3.6 to 8 s^{-1} [Steinmeier and Parkhurst, 1979]). The larger of the two dissociation rate constants (134.6 s^{-1}) is among the largest reported. The CO association rate constant of *H. elongata* is between those of holothurian hemoglobin (*S. briareus*, $\sim$10^3 M^{-1}s^{-1} [Colacino, 1973]; *T. gemmata*, 10^3 to 10^4 M^{-1}s^{-1} [Steinmeier and Parkhurst, 1979]) and human hemoglobins (30 × 10^5 M^{-1}s^{-1} [Antonini and Brunori, 1971]).

Most hemoglobins exhibit a decrease in oxygen affinity as the pH decreases (Weber, 1980). It is generally accepted that most holothurian hemoglobins are insensitive to pH (Terwilliger and Terwilliger, 1988). A weak pH dependence (normal direction) on the oxygen affinity of hemoglobin has only been reported for two holothurians, *Paracauding chilensis* (Baker and Terwilliger, 1993) and *Molpadia arenicola* (Bonaventura *et al.*, 1976). The hemoglobin of *H. elongata*, however, shows a slight increase in its oxygen affinity, both *in cellulo* and *in vitro*, with a decrease in pH (Table 1). The magnitude of the Bohr shift of *H. elongata* hemoglobin,

however, is very small and may not have any adaptive significance under physiological conditions.

There is not a large temperature dependence, as evidenced by the small change in affinity with a change in temperature. The heat of oxygenation, $\Delta H = -4.1$ kcal/mol, is smaller than that reported for other hemoglobins, -6 kcal/mol to -8 kcal/mol (Antonini and Brunori, 1971; Colacino, 1973). The possession of a hemoglobin with a small temperature dependence may be adaptive to an intertidal organism, because it would make it relatively insensitive to the large temperature changes that can occur in the intertidal environment (Cochran and Burnett, 1996; unpubl. data).

The high affinity of the hemoglobin of juvenile brittle stars is clearly different from that of the adults. This functional difference could be adaptive in their normal habitat. The planktonic larvae often settle out onto the extended arms of the adults. The juveniles then crawl down the arm into the burrow of the adult, where they move around within the burrow (pers. obs). Small juveniles (disc diameter < 1 mm) do not extend their arms into the water column as the adults do; therefore, they must obtain oxygen from the burrow environment or from the adult. The measured Po_2 of the burrow water is very low (undetectable with FOXY system [Ocean Optics, Inc.]; unpubl. data), but some oxygen must escape into the environment from the adult, as evidenced by the fact that the sediments lining the burrow are oxidized. The possession of high-affinity hemoglobin by the juveniles would aid them in the acquisition of oxygen from this low-oxygen environment. The switch from high-affinity hemoglobin in the fetus/juvenile to lower affinity hemoglobin in the adult has been documented in many animals (Barcroft, 1935; Riggs, 1951). The time in development in *H. elongata* when the switch from high-affinity to low-affinity hemoglobin occurs is not known. Also unknown is whether the hemoglobin present in the juvenile RBCs is one of the hemoglobins found in the adults or an entirely different one. Due to the small size of the juveniles and the small numbers collected, insufficient hemoglobin has been isolated to make comparisons with adult hemoglobin by gel electrophoresis.

The hemoglobins of *H. elongata* have a higher affinity for oxygen than those of another ophiuroid species, *Ophiactis simplex* (Christensen, 1998) (see Table 1). The differences in the oxygen-binding properties could be related to differences in their lifestyles. *H. elongata* burrows in anoxic mud, does not ventilate its burrow, and lacks genital bursae, structures known to serve as sites of gas exchange in ophiuroids. *O. simplex* is epibenthic and commonly occurs in fouling communities associated with rock jetties and wharf pilings. The oxygen levels in these environments may not be as limiting as in the mud. The function of hemoglobin in *O. simplex* is not known, but is under investigation.

Effects of sulfide on oxygen binding

Vertebrate and many invertebrate hemoglobins bind with sulfide to form sulfhemoglobin. In human hemoglobin A, the binding of sulfide takes place at the heme, but not to the iron, and is effectively irreversible (Berzofsky *et al.*, 1971; Carrico *et al.*, 1978). This also decreases oxygen affinity so much as to render the hemoglobin functionless under normal conditions (Carrico *et al.*, 1978). Many invertebrate hemoglobins show reversible binding with sulfide (Arp and Childress, 1983; Childress *et al.*, 1984; Doeller *et al.*, 1988; Somero *et al.*, 1989). In many cases the binding of sulfide is vital to the organism because it harbors endosymbionts that require sulfide as an energy source (Felbeck, 1983; Hand and Somero, 1983; Powell and Somero, 1986). No such endosymbionts have been observed in *H. elongata* (unpubl. data.)

No abnormal spectral changes were observed during the oxygen equilibrium experiments on *H. elongata* hemoglobin exposed to sulfide. This indicates that either the hemoglobin is insensitive to sulfide or there are detoxifying enzymes present. However, due to the nature of the hemolysate solution, any enzymes present would be greatly diluted, thus decreasing their efficiency. The lack of abnormal spectra points toward a hemoglobin that is insensitive to sulfide. The hemoglobins of the phoronid *Phoronis architecta* (Vandergon and Colacino, 1991) and the polychaete *Abarenicola affinis* (Wells and Pankhurst, 1980) also appear to be insensitive to sulfide. If sulfide does bind to the hemoglobin of *H. elongata*, it does so in a way that does not alter oxygen affinity or the visible absorption spectra. For organisms that live in sulfide-rich environments but do not rely on sulfide-requiring symbionts, it would be highly adaptive to possess hemoglobin whose function is not disrupted by exposure to sulfide.

Summary

It appears that the hemoglobins of *Hemipholis elongata* are well suited to the habitat and lifestyle of the animal. *H. elongata* does not ventilate its burrow, and its aerobic metabolism must be supported by circulation of the WVS fluid and RBCs between arms exposed to the water column and buried body parts. The moderate P_{50} (11.4 mmHg, pH 8.0, at 20 °C) and cooperativity (Hill number $\geq$ 1.7) of the hemoglobin would allow it to extract oxygen from the overlying water column and deliver it to buried body parts over a wide range of external and internal P_{O_2} values. The relative insensitivity of the hemoglobins to changes in temperature and pH preserve hemoglobin function when conditions change, as they frequently do in an intertidal environment. The insensitivity to hydrogen sulfide ensures that the hemoglobins continue to function below the sediment surface where the animal is situated and sulfide levels may be high.

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